IgA Nephropathy in Mice Following Repeated Administration of Conjugated Haemophilus Influenzae Type B Vaccine (PRP-T)

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Objective: In this study, the risk of IgA nephropathy in Swiss albino mice following the subcutaneous administration of conjugated Haemophilus influenzae type b vaccine (PRP-T), containing capsular polysaccharide of the organism (PRP) conjugated to tetanus protein (T), was evaluated.

Methods: Three treatment and corresponding control groups, each containing mice, were constituted and given 2, 4, 6 injections of 1/4 HD of PRP-T or placebo, respectively, at 2-week intervals. All mice in each treatment group were sacrificed two weeks from the last injection to examine sequential glomerular changes.

Results: The histologic examination of renal tissues revealed mesangial proliferation (6/7; 85%) in the first group given 2 doses of vaccine; mesangial proliferation (5/7; 72%) and increase in matrix (7/7; 100%) in the second group given 4 doses; and mesangial proliferation (7/7; 100%), increase in matrix (7/7; 100%), IgA (7/7; 100%) and C3 (3/7; 42%) deposition within mesangium in the third group given 6 doses. No histopathological changes were detected in the renal tissues of any control mouse.

When the experimental groups were compared statistically with their respective controls at the light microscopic level, mesangial proliferation in the first group (p: 0.0047), mesangial proliferation (p: 0.021) and increase in matrix (p: 0.001) in the second group, mesangial proliferation (p: 0.001) and increase in matrix (p: 0.001) in the third group were determined to be significantly different. When study and control groups were compared by immunofluorescence microscopy, only the third group revealed a statistically significant difference with respect to IgA deposition (p: 0.001). C3 deposition was also demonstrated in this group, but it was not significantly different (p: 0.192). However, in no instance was a control mouse found to have any form of immune deposition.

Conclusion: We concluded that conjugated Haemophilus influenzae type b vaccine, given at two-week intervals to a total of six doses, caused secondary IgA nephropathy in mice.

INTRODUCTION

IgAN is the most common glomerular disease worldwide. It is the major cause of the end stage renal disease (1). The diagnosis of IgAN can be established only via histopathologic findings showing diffuse IgA deposition in the glomerular mesangium together with various degrees of focal or diffuse mesangial proliferation in the absence of any systemic disease like Henoch Schoenlein vasculitis or systemic lupus erythematosus (2). IgG, IgM, C3, and terminal complement components, other than IgA, might also be deposited, although less in frequency and intensity, in the mesangium (1, 2).

The pathogenesis of IgAN is poorly understood. However, since there is a temporal association between clinical findings and either infections or antigenic challenge at mucous membranes, the disease is considered to be a

ABBREVIATIONS

Hib Haemophilus influenzae type; PRP Polyribosyl-ribitol-phosphate; purified capsular polysaccharide extracted from Hib; PRP-T PRP conjugated to tetanus protein; IgAN IgA nephropathy; HD Human dose; DIF Direct immunofluorescence
glomerulonephritis related to inflammation of mucosal surfaces. Glomerular injury is thought to result from the formation of nephritogenic antigen-IgA antibody complexes. This hypothesis is supported by the demonstration of cytomegalovirus, Epstein-Barr virus, hepatitis B virus, herpes simplex virus, Escherichia coli O and K, gluten and soy bean antigens deposited in the renal glomeruli of patients with IgAN. Therefore, IgAN is considered to result from an unregulated mucosal immune response to environmental antigens to which the host is chronically exposed. It is also claimed that, in mucosal and peripheral blood lymphocytes of patients with IgAN stimulated by such antigens, the synthesis of IgA antibodies and its polymers is increased (3, 4).

Following the intraperitoneal administration of gram-negative bacteria (including Hib) or their cell wall components, IgA and C3 have been shown to deposit in the glomerular mesangium of mice (4).

In this study, the risk of secondary IgAN in mice due to the administration of an Hib-conjugated vaccine, PRP-T, was evaluated.

MATERIALS AND METHODS

Experimental animals, vaccines, and urinalysis

Swiss albino mice were supplied by the Experimental Animals Research Laboratory of Dokuz Eylul University.

Hib-conjugated vaccines were supplied by the Pasteur Merieux Serums and Vaccines Company (Act-HIBR: Haemophilus influenzae type b polysaccharide conjugated to tetanus protein; PRP-T). Special mouse metabolism cages were used to collect urine from mice for urinalysis. Testing was via Combulyr10-TestR M urinalysis strips (Boehringer Manheim) and light microscopy.

Method

Three treatment groups and their controls, each containing 7 male mice, 6 wks of age (Swiss albino L. of F5 generation with a homogeneity of 87.5%), were constituted and given 2, 4, or 6 injections of 1/4 HD of PRP-T or placebo (isotonic saline solution equal in volume to the vaccine) respectively, at 2-week intervals via subcutaneous injection in the abdominal region (5) (Table 1). Urine samples from all the mice were analyzed for proteinuria and hematuria at the beginning of the study and weekly thereafter. All the mice were sacrificed under ether anesthesia two weeks after the last injection of vaccine or placebo. The kidneys, liver and lung were extirpated for histological examination. The kidneys were examined both by light and DIF microscopy, while only microscopic evaluation was performed on the other tissues.

Histological Evaluation

All tissue sections were evaluated separately by two pathologists. After removing the kidneys, portions were placed either in phosphate buffered solution or fixed in formalin. On the same day, thin sections from the two kidneys were prepared for DIF evaluation, and the remaining renal tissues formalin fixed. Paraffin blocks were prepared via routine procedures from the formalin fixed lung, liver and renal tissues. Sections 3 μm in thickness were prepared from the kidney and stained with hema-toxylin-eosin, Masson's trichrome, or periodic acid-Schiff stains and examined by light microscopy.

The tissues reserved for DIF staining were

Table 1 Number of mice in treated and control groups and vaccination schedule

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of mice</th>
<th>PRP-T*</th>
<th>Placebo†</th>
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<tbody>
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<td>Group II (two injections)</td>
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<tr>
<td>Group II (four injections)</td>
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<td>Group III (six injections)</td>
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*1/4 human dose (0.125 ml) of influenzae type b capsular polysaccharide conjugated to tetanus protein
covered with cryostat mounting medium (Shandon) and frozen of -70°C by carbon dioxide jet (Shandon). Four µm thick sections from the tissues were deposited on poly-L-lysine (Sigma) coated slides. After overnight fixation, sections were further fixed in acetone for five minutes at 4°C, then hydrated in phosphate buffered solution for another five minutes. Following these steps, sections were exposed to fluorescein isothiocyanate-conjugated antibodies against IgG, IgA, IgM, and C3 (DAKO-anti human: at dilutions of 1/100, 1/50, 1/100 and 1/100 respectively) for 30 minutes at room temperature. The sections were then washed with phosphate buffered solution for five minutes before being covered with glycerol aqueous mounting medium (Sigma). Mouse lymphoid tissue was used as a positive control for IgG, IgA and IgM, and a renal tissue section previously determined to be positive was used for C3. The localization and amount of immunodeposits were determined by immunofluorescence microscopy.

Statistics
Kruskal Wallis variance analysis was used to evaluate differences between the treatment groups, while the Fisher chi-square test was used to determine the significance of the differences between treated and control groups.

RESULTS

First Group
a) Urinalysis: Neither hematuria nor proteinuria was detected in any control or treatment group throughout the study.

b) Light microscopy: In 85% (6/7) of the treated mice, there was mesangial cell proliferation in both kidneys (Fig. 1), although the mesangial matrix, membranous, tubulointerstitial, and vascular structures were normal. However, focal tubular atrophy was seen in one kidney of a single mouse. The kidneys of the control group were within normal histological limits (Fig. 2). The difference between the treated and control groups with respect to the renal histopathology was statistically significant (p: 0.0047) (Table 2).

c) DIF microscopy results: There were no immune deposits either in the treated or the control mice.

Second Group
a) Urinalysis: Neither hematuria nor proteinuria was found in any of the control or treated mice.

b) Light microscopy: Focal (3/7, 43%) and diffuse (2/7, 28%) mesangial proliferation and focal (2/7, 28%) and diffuse (5/7, 72%) increases in mesangial matrix were detected in the

Fig. 1 Mesangial proliferation and slight increase in mesangial matrix (from the second group of treated mice, PAS; x400). Mice received 4 incubations of H. influenzae vaccine (PRP-T) at intervals of two weeks. PAS; ×400.
renal tissues of treated mice. The kidneys of the control mice were normal histologically and the difference between the groups was statistically significant (p: 0.021 and p: 0.001 with respect to mesangial proliferation and increase in mesangial matrix). Focal tubular atrophy was observed in two treated mice. (Table 3).

c) DIF microscopy results: DIF examination did not reveal any immune deposits in either group of mice.

Third Group

a) Urinalysis: Hematuria or proteinuria was not detected in the control mice. One of the treated mice was found to have hematuria (8–10 erythrocytes per high power magnification by light microscopy and + in urinalysis strip) just before sacrifice (two weeks after the sixth vaccine dose). There was no significant difference between the groups concerning hematuria (p>0.95).

b) Light microscopy: While there were focal (5/7, 72%) and diffuse (3/7, 42%) mesangial cell proliferation and a diffuse (7/7, 100%) increase in mesangial matrix, the membranous and tubulointerstitial structures were determined to be normal in the treated mice. Renal tissues of the controls were normal histologically. (Table 4). Light microscopic findings differed significantly between the groups (p: 0.001).

c) DIF microscopy results: While there was

![Fig. 2 A normal glomerular structure (from the first group of control mice, PAS; ×200)](image)

Table 2 Renal histopathology findings in treated mice of group one two inoculations of H. influ vaccine, two week apart light microscopy

<table>
<thead>
<tr>
<th>mouse no</th>
<th>Mesangial cell proliferation*</th>
<th>Increase in mesangial matrix</th>
<th>Focal tubular atrophy</th>
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*When compared with the control group, statistically significant (p<0.0047)
D:Diffuse
no immune deposition in the control mice, in all the treated mice there was IgA deposition in the mesangium focally or diffusely (Fig. 3). IgA deposition was found in all the 50 glomeruli examined in 2 mice (28%), whereas the other mice (5/7, 72%) exhibited IgA deposition in 2 to 44 of the 50 glomeruli (Table 5). IgG and IgM deposition were not seen, whereas 2 mice were found to have C3 deposition in from 3 to 14 of the 50 glomeruli examined (Fig. 4). C3 deposition was bilateral in two mice, and unilateral in one mouse (Table 5). With respect to the IgA deposition, the treatment group differed significantly from the control group (p: 0.001). However, C3 deposition was not found to be significantly different between the groups (p: 0.192).

When the light microscopic findings of all three treatment groups were compared with each other no statistically significant differences were found (p: 0.329, SD: 2, KW: 2.222).

By light microscopy, the liver and lung were within normal limits in all the control and treated mice.

**DISCUSSION**

Various procedures have been tried to develop experimental IgAN. Intraperitoneal injection of dextran or apoferritin, intravenous inoculation of an IgA-dinitrophenyl immune complex, and the oral administration of heterologous antigens, performed in mice, are

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Table 3 Renal histopathology findings in treated mice of group two 4 inoculations of H. influenzae vaccine, at 2-wk intervals by light microscopy

<table>
<thead>
<tr>
<th>mouse no</th>
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<th>Focal tubular atrophy</th>
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*When compared with the control group, statistically significant (p<0.021)
†When compared with the control group, (p<0.021)
F:Focal
D:Diffuse

Table 4 Renal histopathology findings in treated mice of group three 6 inoculations of H. influenzae vaccine at 2-wk intervals light microscopy

<table>
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<tr>
<th>mouse no</th>
<th>Mesangial cell proliferation*</th>
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*When compared with the control group, statistically significant (p<0.001)
F:Focal
D:Diffuse
among these procedures (4). Likewise, to develop a passive IgAN, pneumococcal C polysaccharide or phosphorylcholine was used as antigen, while the antiphosphorylcholine IgA of a mouse plasmocytoma was used as antibody. IgA and C3 deposition in the glomeruli of mice has been demonstrated following the simultaneous administration of these antigens intravenously and antibodies intraperitoneally. In this latter model, the pathogenesis of the glomerular injury was reported to depend more on C3 than on IgA (6). Endo et al. (4) reported that the intraperitoneal administration of Gram-negative bacteria (including Hib), or their cell wall components led to the accumulation of IgA and C3 in the glomeruli of mice. Immune deposition was reported to be less intense when the same bacteria were administered orally. It was also reported that cell wall components other than lipopolysaccharides played a more important role in immune deposition. Specific IgA antibody against the PRP

Fig. 3  Granular IgA deposition in the mesangium (from the third group of treated mice, DIF IgA antibody; ×400).
Describe exactly what the third group of mice received. (See Fig. 1)

Fig. 4  Granular C3 deposition in the mesangium (from the third group of treated mice, DIF C3 antibody; ×200)
Here as well, a description of what the third group of mice received (6 inoculations of vaccine at 2-week intervals) is important
and T components of the vaccine were not detected in the plasma or glomeruli of the mice. However, the amount of IgA and C3 deposited in the glomeruli was demonstrated to be in direct correlation with the level of specific plasma IgA antibody to the Gram-negative bacteria administered intraperitoneally in the previous study (4). Furthermore, an experimental IgAN secondary to the administration of Clostridium tetani or tetanus toxoid has not been reported yet.

Although electron microscopy was not performed, the renal histopathological findings obtained via light and DIF microscopy were adequate in making the diagnosis of IgAN (1).

The presence of IgA in the glomerular mesangium as the sole or predominant immunoglobulin is the diagnostic immunopathological pattern of IgAN (2). The present study demonstrates that glomerular deposition of IgA and C3 can be induced in Swiss albino mice by the subcutaneous injection of PRP-T. Mesangial IgA deposition was demonstrated in all 7 mice in the third treatment group, whereas only 3 of the 7 mice (42%) were shown to have C3 deposition. Although IgA deposition is usually coupled with C3 accumulation in IgAN, the absence of C3 deposition in the presence of IgA antibody in the glomerular mesangium does not rule out the diagnosis of IgAN (1, 2). Based on this, all the mice in our third treatment group were considered as IgAN. It is also obvious that the cell wall components of Hib are capable of inducing this immune deposition, indicating that the entire microorganism is not necessary. These findings are in accordance with a previous report (4).

Focal tubular atrophy, one of the extraglomerular histopathological lesions of IgAN (2), was demonstrated in three mice; one in the first and the others in the second study groups.

When the mice were evaluated with respect to hematuria and proteinuria, only one animal in the third treatment group was demonstrated to have hematuria. This findings, therefore, can be regarded as a coincidence.

Histopathological findings obtained by light microscopy were scored as (+) or (++), according to the degree of intensity. The relation between the intensity of the histopathological lesions and the number of vaccine doses was not compared statistically, since histopathological scoring could not be done objectively.

The spontaneous accumulation of immunoglobulins and complement components in the glomeruli of various mouse species has been reported (4). In our study, no immune deposition was demonstrated in the glomeruli of the control mice. It was also reported that glomerular changes due to latent viral infections could occur, especially in aged mice, and spontaneous IgA deposition in the glomeruli can be seen in mice aged 30 weeks or older (4). For that reason, our experiments began with six-week old mice and ended when the oldest mice were 18 weeks of age.

Determination of vaccine doses and vaccination intervals were based on previously reported experiments. Protective levels of antibody formation was demonstrated when the mice were given 1/4 HD of conjugate Hib vaccine; two inoculations two-weeks apart (5). Thus, we did not think it was necessary to determine plasma protective antibody titers after the vaccination schedule had been completed.

Only mesangial cell proliferation and/or mesangial matrix increase without IgA deposition were demonstrated in the first and second groups of treated mice. Mesangial cells have an important role in the clearance of immune complexes (7). Experimental glomerular injury due to injection of a nephrotoxic antigen may resolve completely after a single dose. However, with more than one injection, progressive mesangial cell proliferation, an increase in mesangial matrix, and eventually glomerulosclerosis development may occur (8). Mesangial cell proliferation without an increase in the mesangial matrix was seen in the first group of treated mice. The mesangial proliferation with an increase in matrix which was seen in the second treatment group indicated that the increase in mesangial matrix was related to the increase in antigenic stimulation. The absence of IgA and C3 in the glomeruli of the first and second groups of treated mice can be explained by the clearance of these immune complexes via mesangial cells during this peri-
od. Mesangial proliferation and the increase in matrix, in spite of the absence of immune deposition, may be considered a result of the increased clearance activity of mesangial cells. Therefore, the increased deposition of immune complexes in the glomeruli, secondary to the increased antigen-antibody load due to the ongoing vaccine administration, might have exceeded the clearance capacity of the mesangial cells and thus demonstrable IgA deposition would occur in the mesangium.

Since various pathways leading to secondary IgAN have been defined (1, 2), extrarenal tissues, such as liver and lung, were evaluated histologically. No pathological findings occurring with, or leading to, the changes in the renal tissues could be demonstrated.

Experimental IgAN related to Hib conjugate vaccine(s), or IgAN as a complication of the routine use of these vaccines or natural Haemophilus influenzae type b infection, has not been reported yet. However, IgAN is thought to occur more frequently in children with Haemophilus parainfluenzae colonization of their pharyngeal mucosa (3).

Conjugate Hib vaccines have been reported to decrease, but not totally eliminate, Hib colonization in children (9). It is not clear whether there is a prompt anamnestic response following natural exposure to this microorganism in children administered conjugate Hib vaccines.

In conclusion, this study demonstrated that six inoculations of Haemophilus influenzae type b conjugate vaccine (PRP-T), at 1/4 HD, resulted in secondary IgA nephropathy development in Swiss albino mice. However, where natural Hib infections during routine vaccination, before immunization has been completed, creates a potential risk for IgAN in children needs further evaluation.

REFERENCES